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Functional Analysis of a Dominant Negative Mutant of $G\alpha_{i2}$*

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The key event in receptor-catalyzed activation of heterotrimeric G proteins is binding of GTP, which leads to subunit dissociation generating GTP-bound α subunits and free $\beta\gamma$ complexes. We have previously identified a mutation that abolished GTP binding in $G\alpha_o$ (S47C) and demonstrated that the mutant retained the ability to bind $\beta\gamma$ and could act in a dominant negative fashion when expressed in *Xenopus* oocytes (Slepak, V. Z., Quick, M. W., Aragay, A. M., Davidson, N., Lester, H. A., and Simon, M. I. (1993) *J. Biol. Chem.* 268, 21889–21894). In the current work, we investigated the effects of the homologous mutant of $G\alpha_{i2}$ (S48C) upon signaling pathways reconstituted in transiently transfected COS-7 cells. We found that expression of the $G\alpha_{i2}$ S48C mutant prevented stimulation of phospholipase C (PLC) β2 by free $\beta\gamma$ subunit complexes. This effect of $G\alpha_i$ S48C was not readily reversible in contrast to the inhibitory effect of wild-type $G\alpha_{i2}$, which could be reversed upon activation of the cotransfected muscarinic M2 receptor, presumably by release of $\beta\gamma$ from the G protein heterotrimer. Coexpression of $G\alpha_i$ S48C or the wild-type $G\alpha_{i2}$ also dramatically decreased G_{16}-mediated stimulation of PLC by C5a in the cells transfected with cDNAs encoding C5a receptor and $G\alpha_{16}$. Activation of PLC via endogenous G_q or G_{11} in the presence of α1C adrenergic receptors was similarly attenuated by coexpression of $G\alpha_i$ or $G\alpha_i$ S48C. Pertussis toxin treatment of the transfected cells enhanced the inhibition of the receptor-stimulated PLC by wild-type $G\alpha_i$ subunits but did not influence the effects of the dominant negative mutant. The enhancement of the wild-type $G\alpha_i$ inhibitory effect by pertussis toxin can be explained by stabilization of $G\alpha_i$ binding to $\beta\gamma$ as a result of ADP-ribosylation, while $G\alpha_i$ S48C mutant binds $\beta\gamma$ irreversibly even without pertussis toxin treatment. Therefore, a feasible mechanism to rationalize the attenuation of the $G\alpha_{16}$ and $G_{q/11}$-mediated activation of PLC by cotransfected $G\alpha_i$ is the competition between $G\alpha_i$ and $G\alpha_{16}$ or $G_{q/11}$ for the $\beta\gamma$ complexes, which are necessary for the G protein coupling with receptors. These experiments provide new evidence for the role of $\beta\gamma$ in the integration of signals controlling phosphoinositide release through different $G\alpha$ families.

Many receptors of hormones, neuromediators, and growth factors transmit signals via heterotrimeric G proteins. Receptor-induced activation leads to dissociation of G protein subunits, generating α subunits charged with GTP and free $\beta\gamma$ complexes. In recent years, G protein-mediated signaling has proven to be far more complex than just a combination of "linear" pathways from specific receptors to their effectors. The number of cloned genes encoding G protein subunits, effectors, and receptors includes hundreds of members (1–4). Furthermore, in many signaling pathways, activity of effectors is modulated not solely by $G\alpha$ subunits as was traditionally thought but also by $G\beta\gamma$ complexes (5–11). One of the ways to define the specific links within the network of G proteins, multiple receptors, and effectors is by blocking the signaling circuits by expressing dominant negative mutants of different $G\alpha$ subunits *in vivo*. Mutations of glycine residues in the conservative sequence DVGGQR of the $G\alpha$ subunits were found to reduce GTP binding and activation of the G proteins. Expression of these mutants inhibited pathways controlled by $G\alpha_s$ and $G\alpha_i$ (12–15). We identified another interesting mutation that abolished GTP binding in $G\alpha_o$-$G\alpha_o$ S47C and demonstrated that the mutant retained the ability to bind $\beta\gamma$ and could suppress G protein-mediated signal transduction in *Xenopus* oocytes (16).

In the current work, we studied the effects of expression of the $G\alpha_{i2}$, $G\alpha_{oA}$, and their dominant negative mutants $G\alpha_i$ S48C and $G\alpha_o$ S47C on reconstituted signaling pathways in transiently transfected COS-7 cells. We found that $G\alpha_i$ can attenuate activation of PLC[1] by hormone receptors coupled to the enzyme via members of the G_q family and that the most likely molecular mechanism for this inhibition is competition for $\beta\gamma$ subunits. Our data provide further evidence for the interdependence of G protein-mediated signaling pathways and the important role of $\beta\gamma$ subunits in controlling these interactions.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis and Construction of COS Cell Expression Vectors—The $G\alpha_i$ S48C, $G\alpha_q$ S47C, and $G\alpha_{16}$ S56C mutations were introduced by using polymerase chain reaction (17). The cDNAs were inserted into pCMV vector as previously described (18). The $G\alpha_o$ S47C mutant was subcloned into pCMV from $pG_o\alpha$ bacterial expression vector (16). Preparation of pCMV vectors containing the cDNAs for α1C adrenergic and C5a receptors, PLC β2, and G protein α, β, and γ subunits was previously described (18, 19).

Cell Culture and Transfection—COS-7 cells were maintained in DMEM containing 10% FCS. 1×10^5 cells/well were seeded in 12-well plates 1 day before transfection. The total amount of DNA in all transfections was 1.0 μg/well. The amount of each type of DNA in each set of experiments was equal, and pCIS encoding β-galactosidase was used to maintain a constant amount of DNA. To each well, 1.0 μg of DNA mixed with 10 μl of lipofectamine (Life Technologies, Inc.) in 0.5 ml of Opti-MEM (Life Technologies, Inc.) was added, and 5 h later, 0.5 ml of 20% FCS in DMEM was added to the cells. Cells were assayed for inositol phosphate levels or harvested for protein expression analysis 48 h after transfection.

Analysis of Phosphoinositide Release—1 day post-transfection, the medium was removed, and the cells were washed with phosphate-buffered saline and incubated in 0.4 ml of medium of inositol-free DMEM with 10% dialyzed FCS containing 10 μCi/ml *myo*-[2-^{3}H]inositol (DuPont NEN). 24 h later, the cells were washed with phosphate-buffered saline, 200 μl of inositol-free medium containing 10 mM LiCl was added, and the cells were incubated for 25 min at 37 °C. Each reaction was stopped by adding 200 μl of ice-cold mix of 10% perchloric

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[1] The abbreviations used are: PLC, phosholipase C; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum.

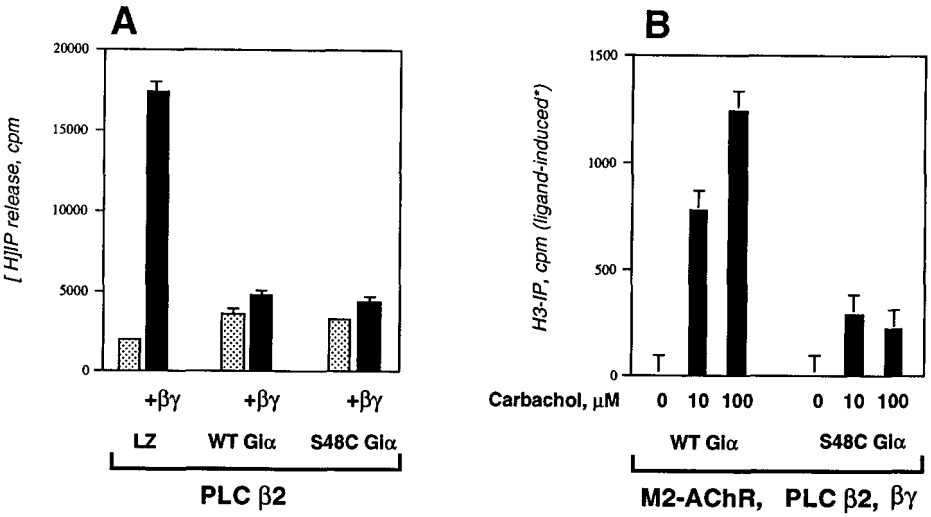

FIG. 1. **Influence of Gα$_i$ and its S47C mutant on stimulation of PLC β2 by free βγ.** *A*, cells were cotransfected with cDNAs encoding phospholipase β2 (0.2 μg), Gβ subunit ($β_1$, 0.2 μg), Gγ (0.2 μg) (γ2 or γ5 were used in different experiments), and wild-type (*WT*) or mutant $Gα_{i2}$ (0.3 μg). Total amount of DNA was adjusted to 1.0 μg by CMV-LZ cDNA. Cells were then labeled with [^{3}H]inositol, and levels of inositol phosphates were determined. Transfection, labeling, and analysis of inositol phosphate release were performed as previously described (15). *B*, cells were cotransfected also with cDNA for muscarinic M2 receptor, and levels of released inositol phosphates were determined after incubation of the cells with indicated concentrations of carbachol.

and 0.2% phytic acids and incubating the cells on ice for 10 min. Then, 200 μl of the supernatant was transferred to a microcentrifuge tube and neutralized with 2 M KOH. After centrifugation, the supernatant was loaded on 0.5 ml of AG1-X8 anion exchange column (200–400 mesh, formate form, Bio-Rad) separated and counted as described earlier (18, 19). All the data represent duplicate determinations in a single experiment. The error bar expresses the range of the duplicates. Three additional experiments gave similar results.

Pertussis Toxin Labeling—The membranes were prepared from the transfected COS cells as described (19). Membranes (30 μl, 1–2 mg/ml protein) were preincubated with guanine nucleotides for 15 min at 22 °C, and then [^{32}P]NAD (5 μl, 0.1 μM, 500,000 cpm) and activated pertussis toxin (5 μl, 10 ng) was added. After 30 min of incubation, the samples were mixed with 10 μl of a 5 × SDS-polyacrylamide gel electrophoresis sample buffer, heated for 3–5 min at 95 °C and analyzed on 10% polyacrylamide gel as previously described (20).

RESULTS AND DISCUSSION

Substitution of serine 47 for cysteine in $Gα_o$ abolished GTP binding, but the mutant retained its interaction with βγ. Due to the apparent inability to release βγ upon the hormonal activation, this mutant behaved as dominant negative in the G_o-mediated signaling pathway reconstituted in *Xenopus* oocytes (16). To obtain a similar mutant of Gα$_i$, we replaced the homologous Ser-48 with Cys by modifying the cDNA of $Gα_{i2}$. We tested this mutant to determine if it would inhibit pertussis toxin-sensitive pathways of PLC stimulation in mammalian cells. In these pathways, PLC is apparently activated by free βγ complexes (8–10) that are released from G protein heterotrimers upon hormonal stimulation. Fig. 1 demonstrates that in the COS-7 cells cotransfected with cDNAs for β, γ, and PLC β2, there is a 4–6-fold stimulation of phosphoinositide hydrolysis compared with cells expressing PLC β2 alone. Cotransfection of both wild-type Gα$_i$ cDNA and the Gα$_i$ S48C mutant abolished the βγ-induced PLC activity, apparently due to sequestration of free βγ. However, the behavior of mutant and the wild-type Gα subunits was different with respect to receptor-mediated activation of PLC. In the cells cotransfected with muscarinic M2 receptors (Fig. 1*B*), carbachol stimulation resulted in an increase of inositol phosphates released in the presence of βγ and wild-type Gα$_i$. In contrast, no ligand-induced activity was observed in cells cotransfected with the S48C mutant of Gα$_i$, presumably because it bound βγ irreversibly.

PLC β2 can be stimulated by two pathways: pertussis toxin-sensitive (by βγ subunits released upon the activation of G proteins (members of the G_i family)) and pertussis toxin-insensitive (by Gα subunits of the G_q family). If both pathways share the same pool of βγ subunits, the dominant negative mutants of G_i family α subunits would not only block the stimulation of PLC by βγ but also prevent its activation through G_q family α subunits. To test this idea, we cotransfected COS-7 cells with cDNAs encoding the C5a receptor and $Gα_{16}$, reconstituting a pathway for G_{16}-mediated stimulation of endogenous PLC by C5a (Fig. 2). Coexpression of either wild-type $Gα_{i2}$ subunit or its mutant S48C in the system did not have a significant effect on the basal level of inositol phosphate release but markedly reduced the C5a stimulation. Similar inhibitory effects were observed upon cotransfection with other pertussis toxin-sensitive Gα subunits such as $Gα_{i3}$, $Gα_o$, or the $Gα_o$ S47C mutant (data not shown). Western analysis demonstrated that introduction of these proteins did not change the level of $Gα_{16}$ expression (Fig. 2*B*). This suggested, that the inhibitory effect of Gα$_i$ proteins is due to specific interaction with components of the reconstituted signaling pathway and not because of interference with transcription/translation machinery of the transfected cells. The maximal level of inhibition of the C5a receptor-PLC pathway by Gα$_i$ was around 70%. It is possible that the inhibitory effect could have been larger if all of the transfected cells could take up and express all three transfected cDNAs. The effect of Gα$_i$ was proportional to the amount of protein expressed in the cells, *i.e.* higher concentrations of Gα$_i$ caused stronger inhibition of the C5a-induced PLC activity (Fig. 3). This observation suggests that inhibition occurred due to competition between G_i and G_{16} α subunits for the interaction with other protein components involved in the C5a induction of PLC activity.

Both Gα$_i$ and $Gα_{16}$ have been shown to couple to the C5a receptor (21–23). It is possible that Gα$_i$ sequesters βγ, which may be necessary for coupling of C5a receptor with $Gα_{16}$. Alternatively, G_i and G_{16} may compete for interaction with the receptor. Coupling of G_i with its cognate receptors can be abolished after ADP-ribosylation with pertussis toxin. We found, however, that ADP-ribosylation of wild-type Gα$_i$ promoted even further inhibition of the C5a-induced PLC activation, while cells expressing only C5a and $Gα_{16}$ were insensitive to the toxin (Fig. 4). This observation implies that Gα$_i$ attenuates the C5a-induced activation of PLC by competing with the $Gα_{16}$ not on the receptor but rather by competing for βγ. It is known that ADP-ribosylation stabilizes association of the α and βγ subunits (24). In the pertussis toxin-treated cells, the wild-type Gα$_i$ binds βγ stronger and therefore competes with $Gα_{16}$ for βγ more efficiently. Further support for this notion comes from cotransfecting $Gα_{16}$ and C5a receptor together with the S48C mutant of Gα$_i$. Treatment of these cells with pertussis toxin did not lead to any further inhibition because the mutant has been

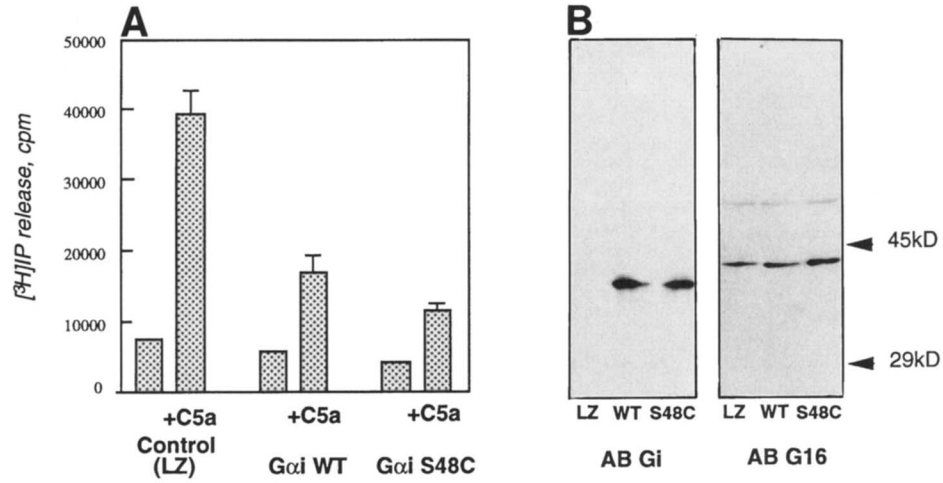

FIG. 2. **Inhibition of C5a-stimulated PLC activity by $G\alpha_i$ and its S48C mutant in transiently transfected COS-7 cells.** Cells were transfected with C5a receptor (0.2 μg) and $G\alpha_{16}$ (0.2 μg) cDNAs together with cDNAs (0.6 μg) corresponding to wild-type (*WT*) $G\alpha_{i2}$, its S48C mutant, or pCMV-LZ (control). *A*, levels of inositol phosphates released were determined with no ligand or in the presence of 0.25 μM C5a. *B* and *C*, Western blot analysis of the transfected COS-7 cells. Cells were treated the same way as those for the PLC assay with exception that [^{3}H]inositol was omitted from the media. On the day of the PLC assay, they were harvested and subjected to Western analysis with antibodies raised against $G\alpha_i$ (*B*) or $G\alpha_{16}$ (*C*).

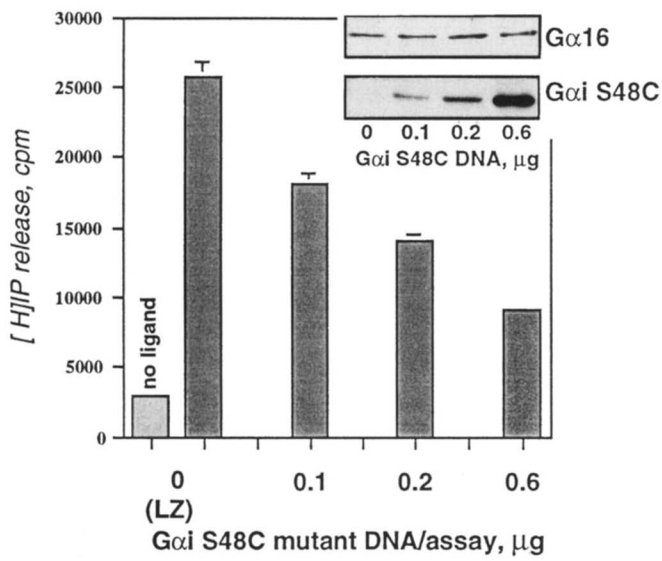

FIG. 3. **Inhibition of C5a-stimulated PLC activity by different amounts of $G\alpha_i$ S48C mutant.** Cells were transfected with C5a receptor (0.2 μg) and $G\alpha_{16}$ (0.2 μg) cDNAs and indicated amounts of $G\alpha_{i2}$ S48C mutant cDNA. The total amount of DNA per transfection was adjusted to 1.0 μg with pCMV-LZ. Levels of inositol phosphates were determined with no ligand (*open bar*) or in the presence of 0.25 μM of C5a (*gray bars*). *Inset*, Western analysis of identically treated cells with antibodies raised against $G\alpha_i$ (*$G\alpha_i$ S48C*) and $G\alpha_{16}$.

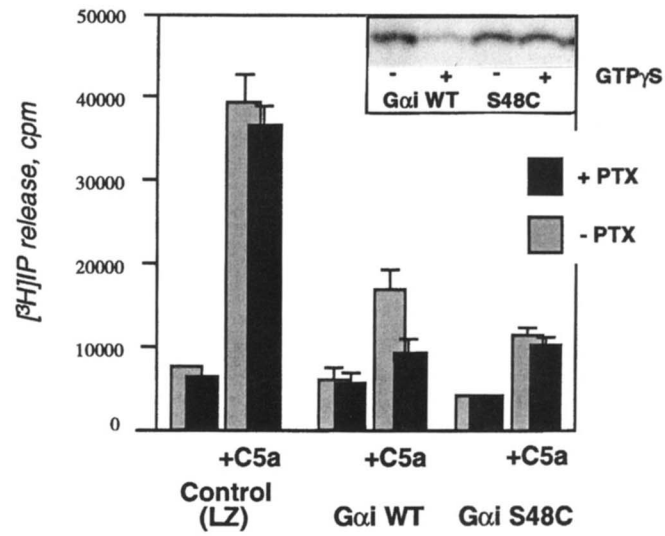

FIG. 4. **Influence of pertussis toxin (*PTX*) on inhibition of C5a-stimulated PLC by $G\alpha_i$ or its S48C mutant in transfected COS-7 cells.** Cells were transfected with C5a receptor (0.2 μg), $G\alpha_{16}$ (0.2 μg) cDNAs and cDNAs (0.6 μg) corresponding to wild-type $G\alpha_{i2}$, its S48C mutant, or pCMV-LZ (control). Levels of inositol phosphates were determined after treatment with (*black bars*) or without (*gray bars*) 200 μg/ml pertussis toxin for 4 h at 37 °C prior to addition of 0.25 μM C5a. *Inset*, influence of 100 μM GTPγS on pertussis toxin-catalyzed ADP-ribosylation of wild-type $G\alpha_i$ and the S48C mutant. Cell membranes were obtained and treated with pertussis toxin in the presence of [^{32}P]NAD, 10 μM GDP, and with or without 0.1 mM GTPγS as described under "Experimental Procedures." Proteins were then resolved by SDS electrophoresis. Gels were stained with Coomassie Blue, dried, and exposed to x-ray film.

shown to bind $\beta\gamma$ irreversibly even without pertussis toxin treatment (13). To ensure that both wild-type and the mutant $G_i\alpha$ were ADP-ribosylated equally, we treated the membrane preparations of the COS-7 cells with pertussis toxin in the presence of [^{32}P]NAD. Fig. 4 (*inset*) demonstrates that both proteins were labeled to a similar extent, and, as in the case with $G\alpha_o$ (13), GTPγS did not influence labeling of the S48C mutant but drastically reduced the modification of the wild-type $G\alpha_i$. These experiments suggest that the mechanism for the attenuation of hormone-activated PLC by $G\alpha_i$ is based upon sequestering of $\beta\gamma$ that can be "shared" with $G\alpha_{16}$. It is unlikely that competition occurs at the effector level because previous evidence argues against the interaction of G_i family proteins with PLC (10, 18, 19). The possibility that $G\alpha_i$ causes the inhibition of PLC indirectly by activation of a different effector is also ruled out because the $G\alpha_i$ dominant negative mutant, which cannot bind GTP and be activated, is more potent in attenuation of hormone-stimulated PLC than wild-type $G\alpha_i$.

If it is binding of $\beta\gamma$ that is responsible for the interference of $G\alpha_i$ with the $G\alpha_{16}$ ($G\alpha_{q/11}$)-mediated signaling, we would expect that $G\alpha_i$ would inhibit ligand induction through receptors that do not interact with $G\alpha_i$, such as α1C adrenergic receptors (25). This was indeed the case; in the cotransfected COS-7 cells, α1C adrenergic receptor-PLC coupling, which is apparently mediated by endogenous $G_{q/11}$ (19), was inhibited by $G\alpha_i$ and $G\alpha_o$ (Fig. 5). As found with the C5a receptor, this inhibition was

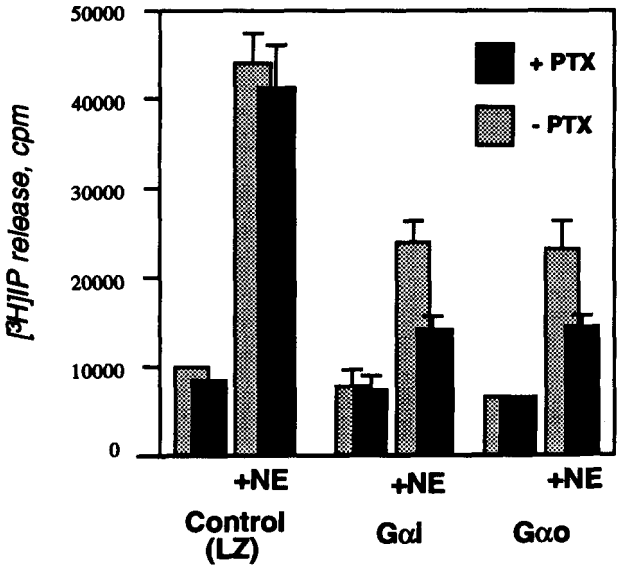

FIG. 5. **Effect of $G\alpha_i$ and $G\alpha_o$ on stimulation of PLC by α1C adrenergic receptor in transiently transfected COS-7 cells.** Cells were transfected with α1C-adrenergic receptor (0.4 μg) cDNA and cDNAs (0.6 μg) corresponding to wild-type $G\alpha_{i2}$, $G\alpha_{oA}$, or pCMV-LZ. Stimulation of inositol phosphate release by 10 μM norepinephrine (*NE*) was determined after (*black bars*) or with no (*gray bars*) treatment with pertussis toxin (*PTX*).

significantly enhanced by pertussis toxin treatment. Therefore, it is quite likely that endogenous $\beta\gamma$ is a limiting factor for the coupling of $G\alpha_{16}$ or $G\alpha_{q/11}$ with their cognate receptors.

Recently published crystal structures of transducin α subunit complexes with GTPγS (26) and GDP (27) show that the hydroxyl group of serine 43, which is homologous to serine 48 in $G\alpha_i$, coordinates with Mg^{2+} found in the GTP binding pocket. The conversion of the –OH to –SH in a Ser → Cys mutant apparently does not cause a major disruption of the overall structure of Gα protein, since $G\alpha_i$ S48C can still bind $\beta\gamma$. Because the serine residue is conserved in the Gα family, we introduced the Ser → Cys mutation into different Gα subunits to use them for inhibition of specific signaling pathways. However, the mutations introduced in $G\alpha_q$ (S47C), $G\alpha_{16}$ (S56C) resulted in a null phenotype (data not shown). The mutants were expressed in cells at the same level as wild-type proteins according to Western analysis but failed to reveal any functional activity. They did not stimulate PLC and did not inhibit hormone-stimulated PLC or the stimulation of the enzyme by free $\beta\gamma$ complexes. It is noteworthy that introduction of other putative dominant negative mutations, G203T and G204A, into $G\alpha_q$ and $G\alpha_{11}$ also resulted in a null phenotype,[2] while the homologous mutations G203T and G204A in G_i (12–15) or G_o (16) proved to be functional. Interestingly, mutants G203T and G204A of recombinant $G\alpha_o$ had very similar biochemical properties, yet only G203T $G\alpha_i$ behaved as a dominant negative *in vivo* while G204A resulted in null phenotype (14). At this point, we do not understand exactly why the mutants of G_q family α subunits were inactive. However, these results suggest that there are local differences in structure and activity even in highly conserved regions of Gα subunits. Detailed comparison of the crystal structures of G_i and G_q α subunits will shed light on the differences between these highly homologous proteins.

Cross-talk between the different G protein-mediated signaling pathways has been previously demonstrated (28–30); for instance, adenylate cyclase type IV was found to integrate signals coming through G_s, G_i, and G_q (31). Here, we demonstrate that $G\alpha_i$-like proteins can alter the pathways regulating PLC via G_q family G proteins. Can this occur *in vivo*? Some indirect evidence supports the existence of such mechanisms. For example, *in vivo* most of the signaling through C5a receptor is pertussis toxin sensitive, whereas *in vitro* C5a receptor couples to pertussis toxin-insensitive G_{16}. In neutrophils where $G\alpha_{i2}$ is the most abundant Gα protein, these contradictory observations can be reconciled if a mechanism similar to the one shown on Fig. 4 took place, *i.e.* ADP-ribosylation increased affinity of $G\alpha_i$ for $\beta\gamma$, thus preventing coupling of receptor to G_{16}. Therefore, the apparent pertussis toxin sensitivity of G_{16}-mediated signaling can be explained as a result of depleting the pathway of $\beta\gamma$. In light of such a possibility, the interpretation of the experiments on pertussis toxin treatment of cells must be done with care; ADP-ribosylation not only can uncouple G_i from its cognate receptor, but it can shift the equilibrium $\alpha_i + \beta\gamma = \alpha i\beta\gamma$ toward the heterotrimer, thus reducing the available pool of $\beta\gamma$ subunits and affecting other pathways. This mechanism implies that $\beta\gamma$ is a limiting factor for G_{16} (G_q)-mediated signaling. Recent data show that $\beta\gamma$ can be bound by other proteins such as effectors PLC β (8–10), PLA2 (6), inositol kinase (32), and K^+ channel (33, 34), *ras*-related proteins (35), phosducin (36), receptor kinase, and other proteins containing pleckstrin homology domains (37, 38), calmodulin (39), etc. Therefore, the GDP-bound Gα subunits compete for the binding to $\beta\gamma$ not only with different Gα but also with these "other" proteins. It is clear that relative affinities of the different Gα subunits for $\beta\gamma$ complexes are critical for specific channeling of signals. Another important notion is that regulation of the expression level of the G protein subunits could provide additional diversity to signal transduction pathways in various cells.

[2] A. Katz, T. Wilkie, and D. Wu, unpublished data.

REFERENCES

1. Gilman, A. G. (1987) *Annu. Rev. Biochem.* **56,** 615–649
2. Birnbaumer, L., Abramowitz, J., and Brown, A. M. (1990) *Biochim. Biophis. Acta* **1031,** 163–224
3. Simon, M. I., Sthrathman, M. P., and Gautam, N. (1991) *Science* **252,** 802–808
4. Birnbaumer, L. (1992) *Cell* **71,** 1069–1072
5. Logothetis, D. E., Kurachi, Y., Galper, J., Neer, E. J., and Clapham, D. E. (1987) *Nature* **325,** 321–326
6. Jelsema, C. L., and Axelrod, J. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 3623–3627
7. Tang, W. J., and Gilman, A. G. (1991) *Science* **254,** 1500–1503
8. Smrcka, A. V., and Sternweis, P. C. (1992) *J. Biol. Chem.* **268,** 9667–9674
9. Camps, M., Carozzi, A., Schnabel, P., Scheer, A., Parker, P. J., and Gierschik, P. (1992) *Nature* **360,** 684–686
10. Katz, A., Wu, D., and Simon, M. I. (1992) *Nature* **360,** 686–688
11. Taussig, R., Iniguez-Liuhi, J. A., and Gilman, A. G. (1993) *Science* **261,** 218–221
12. Hermouet, S., Merendino, J. J., Gutkind, J. S., and Spiegel, A. M. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 10455–10459
13. Osawa, S., and Johnson, G. L. (1991) *J. Biol. Chem.* **266,** 4673–4676
14. Winitz, S., Gupta, S. K., Qian, N.-X., Heasley, L. E., Nemenoff, R. A., and Johnson, G. L. (1994) *J. Biol. Chem.* **269,** 1889–1895
15. Carrel, F., Dharmawardhane, S., and Clark, A. M. (1994) *Mol. Biol. Cell* **5,** 7–16
16. Slepak, V. Z., Quick, M. W., Aragay, A. M., Davidson, N., Lester, H. A., and Simon, M. I. (1993) *J. Biol. Chem.* **268,** 21889–21894
17. Higuchi, R., Krummel, B., and Saiki, R. (1989) *Nucleic Acids Res.* **16,** 7351–7367
18. Wu, D., Lee, C. H., Rhee, S. G., and Simon, M. I. (1992) *J. Biol. Chem.* **267,** 1811–1817
19. Wu, D., Katz, A., Lee, C. H., and Simon, M. I. (1992) *J. Biol. Chem.* **267,** 25798–25802
20. Slepak, V. Z., Wilkie, T., M., and Simon, M. I. (1993) *J. Biol. Chem.* **268,** 1414–1423
21. Amatruda, T., III, Gerard, N. P., Gerard, C., and Simon, M. I. (1993) *J. Biol. Chem.* **268,** 10139–10144
22. Kikuchi, A., Kozawa, O., Kaibuchi, K., Katada, T., Ui, M., and Takai, Y. (1986) *J. Biol. Chem.* **261,** 11558–11562
23. Cowen, D. S., Baker, B., and Dubyak, G. R. (1990) *J. Biol. Chem.* **265,** 16181–16189
24. Huff, R. M., and Neer, E. J. (1986) *J. Biol. Chem.* **261,** 1105–1110
25. Schwinn, D. A., Page, S. O., Middleton, J. P., Lorenz, W., Liggeff, S. B., Yamamoto, K., Lapetina, E. G., Caron, M. G., Lefkowitz, R. J., and Cotecchia, S. (1991) *Mol. Pharmacol.* **40,** 619–626
26. Noel, J. P., Hamm, H. E., and Sigler, P. B. (1993) *Nature* **366,** 654–663
27. Lambright, D. G., Noel, J. P., Hamm, H. E., and Sigler, P. B. (1994) *Nature* **369,** 621–628

28. Kanaho, Y., Tsai, S. C., Adamik, R., Hewlett, E. L., Moss, J., and Vaughan, M. (1984) *J. Biol. Chem.* **259,** 7378–7381
29. Federman, A. D., Conklin, B. R., Schrader, K. A., Reed, R. R., and Bourne, H. R. (1992) *Nature* **356,** 159–161
30. Ashkenazi, A., Winslow, J. W., Peralta, E. G., Peterson, G. L., Schimerlik, M. I., Capon, D. J., and Ramachandran, J. (1987) *Science* **238,** 672–675
31. Lustig, K. D., Conklin, B. R., Herzmark, P., Taussig, R., and Bourne, H. R. (1993) *J. Biol. Chem.* **268,** 13900–13905
32. Stephens, L., Smrcka, A. V., Cooke, F. T., Jackson, T. R., Sternweis, P. C., and Hawkins, P. T. (1994) *Cell* **77,** 83–93
33. Wickman, K. D., Iniguez-Liuhi, J. A., Davenport, P. A., Taussig, R., Krapivinsky, G. B., Linder, M. E., Gilman, A. G., and Clapham, D. E. (1994) *Nature* **368,** 255–257
34. Reuveny, E., Slesinger, P. A., Inglese, J., Morales, J. M., Iniguez-Liuhi, J. A., Lefkowitz, R. J., Bourne, H. R., Jan, Y. N., and Jan, L. Y. (1994) *Nature* **370,** 143–146
35. Crespo, P., Xu, N., Simonds, W. F., and Gutkind, J. S. (1994) *Nature* **369,** 418–420
36. Bauer, P. H., Muller, S., Puzicha, M., Pipig, S., Helmreich, E. J. M., and Lohse, M. J. (1992) *Nature* **358,** 73–76
37. Pithcer, J. A., Inglese, J., Higgins, J. B., Arriza, J. L., Casey, P. J., Kim, C., Benovic, J. L., Kwatra, M. M., Caron, M. G., and Lefkowitz, R. J. (1992) *Science* **257,** 1264–1267
38. Koch, W. J., Hawes, B. E., Inglese, J., Luttrell, L. M., and Lefkowitz, R. J. (1994) *J. Biol. Chem.* **269,** 6193–6197
39. Katada, T., Kusakabe, K., Oinuma, M., and Ui, M. (1987) *J. Biol. Chem.* **262,** 11897–11900